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Coordination of glycerol utilization and clavulanic acid biosynthesis to improve clavulanic acid production in *Streptomyces clavuligerus*

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The glycerol utilization (*gyl*) operon is involved in clavulanic acid (CA) production by *Streptomyces clavuligerus*, and possibly supplies the glyceraldehyde-3-phosphate (G3P) precursor for CA biosynthesis. The *gyl* operon is regulated by GylR and is induced by glycerol. To enhance CA production in *S. clavuligerus*, an extra copy of *ccaR* expressed from *Pgyl* (the *gyl* promoter) was integrated into the chromosome of *S. clavuligerus* NRRL 3585. This construct coordinated the transcription of CA biosynthetic pathway genes with expression of the *gyl* operon. In the transformants carrying the *Pgyl*-controlled regulatory gene *ccaR*, CA production was enhanced 3.19-fold in glycerol-enriched batch cultures, relative to the control strain carrying an extra copy of *ccaR* controlled by its own promoter (*PccaR*). Consistent with enhanced CA production, the transcription levels of *ccaR*, *ceas2* and *claR* were significantly up-regulated in the transformants containing *Pgyl*-controlled *ccaR*.

clavulanic acid, *ccaR*, glycerol utilization, *Streptomyces clavuligerus*

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Clavulanic acid (CA) is a non-classical β-lactam compound [1] produced by *Streptomyces clavuligerus*. As a β-lactamase inhibitor, it is widely used in combination with conventional β-lactam antibiotics, such as penicillin, to treat bacterial infections. The clinical importance of CA has generated much interest in the biosynthesis and the biosynthesis regulation of this compound in recent years (reviewed in [2]). *S. clavuligerus* produces three β-lactam antibiotics: CA, 5S clavams, and cephamycin C. CA and 5S clavams share an early biosynthetic pathway that diverges after the first common intermediate, clavaminic acid (Figure 1). Three distinct gene clusters involved in CA and 5S clavam biosynthesis have been identified, designated the CA, the clavam and the paralog gene clusters. The CA and clavam clusters situate on the chromosome, whereas the paralog cluster resides on the pSCL4 plasmid. The CA and cephamycin C clusters are located in contiguous regions on the chromosome to form a super cluster. The biosynthesis of CA and cephamycin C is co-regulated by a primary regulator CcaR, which is a member of the *Streptomyces* antibiotic regulatory protein (SARP) family [3]. The coding gene of CcaR (*ccaR*) locates in the cephamycin C gene cluster next to *cmcH* (Figure 2A). By binding to a 915 bp intergenic (*cmcH-ccaR*) region, CcaR regulates its own expression. It also binds to the *cefF*, *lat*, and *cefD-cmcI* promoter regions in the cephamycin C gene cluster and to the *ceas2*, *claR* and *oppA1* promoter regions in the CA gene cluster [4] (Figure 2A). Therefore, CcaR positively regulates a large portion of genes in the CA biosynthetic gene cluster by directly con-

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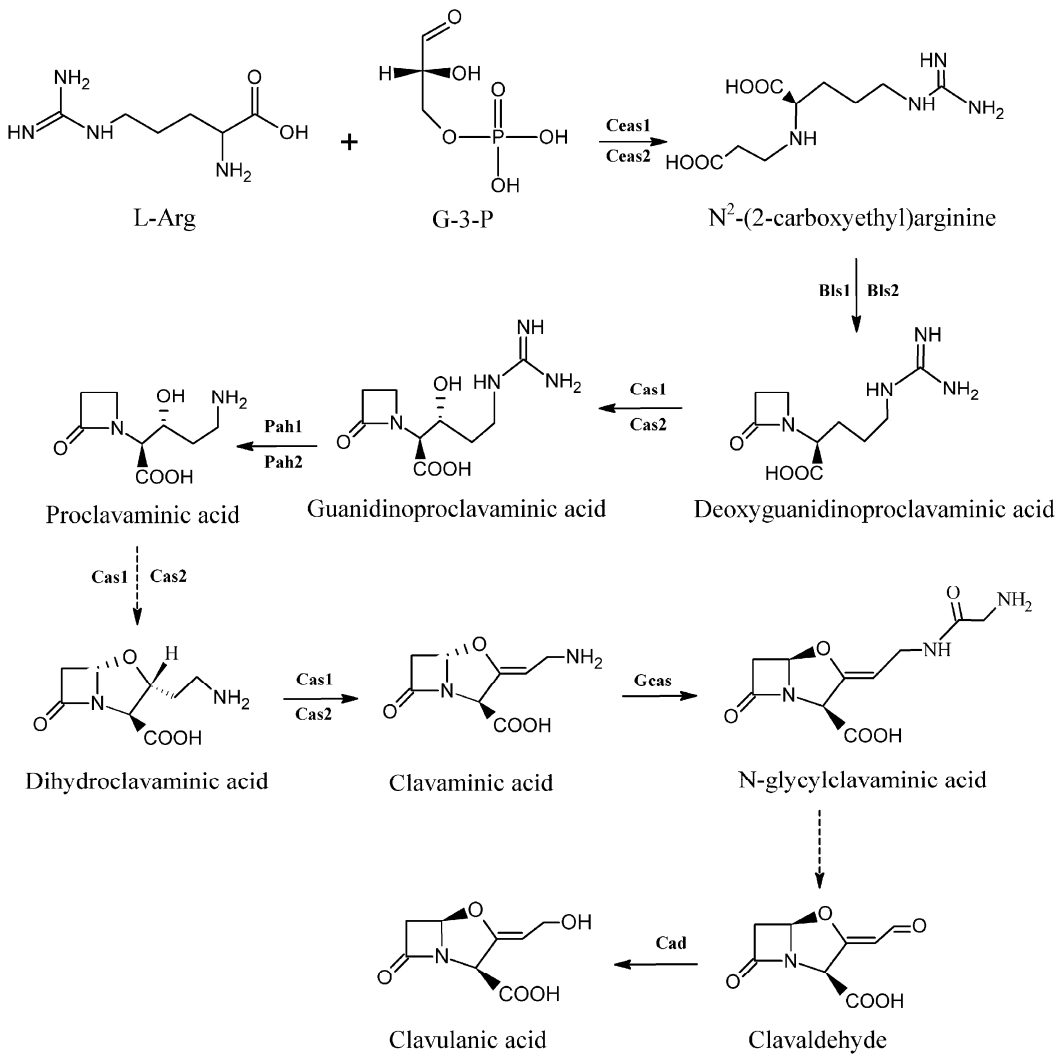

Figure 1 The CA biosynthetic pathway in *S. clavuligerus*. The solid and broken lines represent known and unknown steps in the pathway, respectively.

trolling the transcription of *ceas2, claR* and *oppA1*. The foremost of these transcripts leads a large polycistronic transcript of several genes involved in the early stages of CA biosynthesis, while the *claR* transcript positively regulates the expression of genes involved in the late steps of CA biosynthesis [5–8]. Over-expression of *ccaR* is a proven effective strategy for improving CA production [9–11].

The promoter region upstream of *ccaR* has two transcription start points (Figure 2B) [12]. Upstream of this promoter region, a butyrolactone receptor binding consensus sequence, designated ARE (autoregulatory element, [13]) has been identified as the binding site of *S. clavuligerus* butyrolactone receptor Brp and an IclR family regulator AreB [14,15]. Interestingly, *ccaR* expression is also regulated by *bldG*, which encodes a putative anti-anti-sigma factor [16]. Very recently, Kurt et al. [17] identified a small messenger RNA (mRNA) in the intergenic *cmcH-ccaR* region, which appears to exert positive effects on *ccaR* expression. These findings clearly show the complexity of *ccaR* regulation.

Since glyceraldehyde-3-phosphate (G3P) is a direct precursor of CA biosynthesis, CA production can be controlled by manipulating G3P supply. Li and Townsend [18] increased G3P supply by targeting the glycolytic pathway. In their study, CA production by a mutant of *gap1*, which encodes one of the G3P dehydrogenases in *S. clavuligerus*, was twice as high as that of the control. G3P may also be supplied by the glycerol utilization pathway. Genes in this pathway constitute a conserved *gyl* operon in Streptomycetes (Figure 3A). The *gyl* operon, first characterized from *S. coelicolor*, is a polycistronic transcript whose several genes are collectively termed *gylCABX*. The first three genes encode a putative glycerol transporter, a glycerol kinase, and a glycerol-3-phosphate dehydrogenase, while *gylX* encodes a gene of unknown function [19,20]. The *gyl* operon is transcribed from two closely spaced glycerol-inducible and glucose-repressible promoters, named *gyl*P1/P2. Glycerol induction and glucose repression of *gyl*P1/P2 are probably mediated by the glycerol-inducible repressor GylR, whose

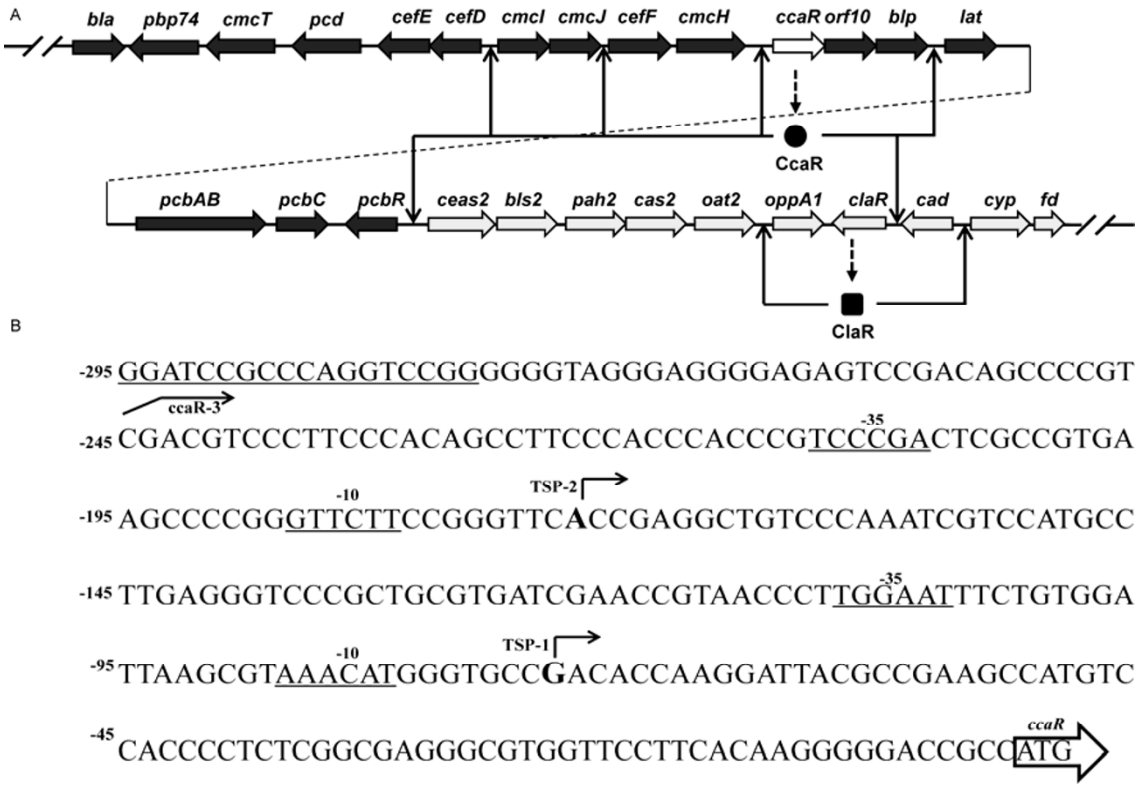

Figure 2 The cephamycin-CA supercluster and promoter region of *ccaR* in *S. clavuligerus*. A, Biosynthesis of cephamycin C (deep gray) and CA (light gray) and the regulatory relationship between related genes. B, Diagrammatic representation of the *ccaR* promoter region. The unfilled arrow represents the *ccaR* coding sequence, including the start codon. TSP-1 and TSP-2 of the *ccaR* transcripts are shown in bold type and the solid arrows represent the mapped *ccaR* transcripts. The -10 and -35 promoter regions are indicated. The forward primer ccaR-3 used to amplify *ccaR* and its promoter is underlined. TSP, transcription start point.

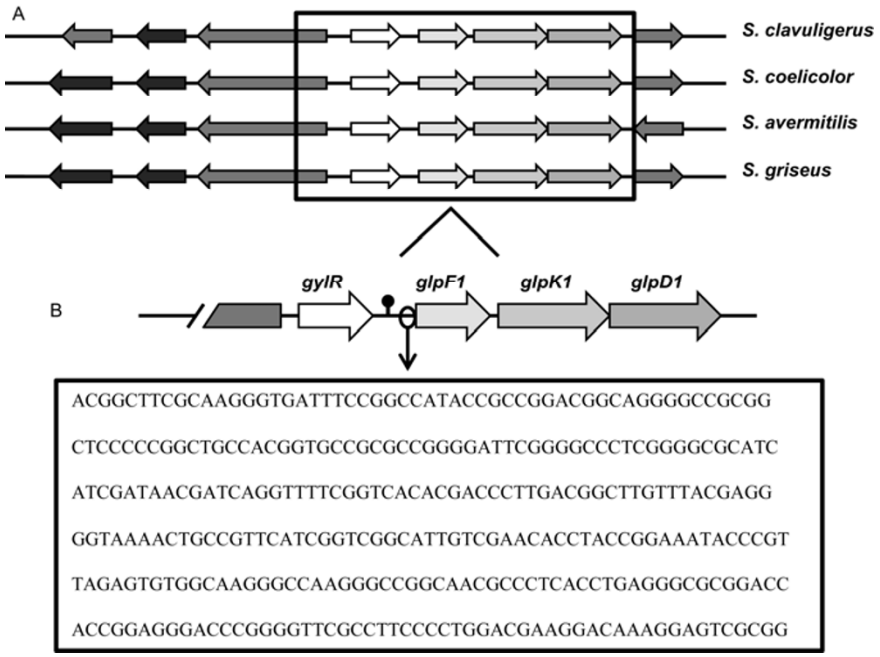

Figure 3 Conservative organization of the glycerol utilization operon in *Streptomycetes*. A, Conservative organization of glycerol utilization operon in *Streptomycetes*. B, Partial enlarged view and annotations of the glycerol utilization cluster in *S. clavuligerus*. The *Pgyl* used in this study is designed to exclude the transcriptional terminator downstream of *gylR*, and the sequences of *Pgyl* are shown below. The transcriptional terminator is also shown.

encoding gene locates immediately upstream of the *gylCABX* operon. GylR controls the expression of both *gylR* and *gylCABX* [21]. In *S. clavuligerus*, the glycerol utilization genes form a similarly organized *gyl* cluster (Figure 3B), designated *gylR-glpF1-glpK1-glpD1* [22]. The *gylR* gene encodes a regulator of the IclR family that is homologous to other GylRs; that is, *glpF1K1D1* are homologous to *gylCAB*. Interestingly, two copies of the *gyl* operon exist in the *S. clavuligerus* genome. The second copies of *glpF2* and *glpK2* cluster together and encode proteins that are 50% and 70% identical to GlpF1 and GlpK1 respectively, while *glpD2* locates elsewhere on the chromosome and encodes a protein that is 32% identical to GlpD1. Whether the second cluster functions in glycerol utilization remains unknown. The importance of the glycerol utilization pathway (*glpF1-K1D1*) in CA production has been demonstrated in *S. clavuligerus* [22]. The expression of the *gyl* operon in *S. clavuligerus* may influence CA production by moderating G3P supply.

Given the importance of glycerol utilization in CA biosynthesis, we have developed an engineering strategy for coordinating glycerol utilization and CA biosynthesis in *S. clavuligerus*. An extra copy of *ccaR* was cloned downstream of *Pgyl* and integrated into the *S. clavuligerus* genome. This construct places both *ccaR* and the *gyl* operon under the control of GylR, enabling their simultaneous induction by glycerol. Our results clearly demonstrate that CA production in glycerol-supplemented batch cultures is improved by this strategy. Consistent with the improved CA titers, expressions of *ccaR*, *ceas2* and *claR* were also significantly increased.

1 Materials and methods

1.1 Strains, plasmids, primers and growth conditions

The strains, plasmids and oligonucleotides used in this study are listed in Tables 1 and 2. Wild-type *S. clavuligerus* NRRL 3585 and its derivatives were maintained on yeast dextrose (YD) agar [23]. Exconjugants were selected in AS-1 medium [24] supplemented with 10 mmol L^{-1} $MgCl_2$. To assess the transcriptional activities of *Pgyl* and *PccaR*, *S. clavuligerus* was cultured in asparagine (SA) media [24]. CA fermentations were carried out in soybean flour medium as described previously [24]; these cultures were sampled for quantitative transcription analysis.

E. coli strain DH5α was used as the host for DNA manipulation as described previously [25]. *E. coli* ET12567/pUZ8002 [26] was used as the conjugation strain for transferring DNA to *Streptomyces* according to the standard protocol [27]. Restriction enzymes, T4 DNA ligase, and KOD DNA polymerase were purchased from Takara (Dalian, China). Primer synthesis and DNA sequencing were performed by Invitrogen (Beijing, China). All chemicals and reagents were purchased from Sigma-Aldrich. 50 μg mL^{-1} of apramycin or 50 μg mL^{-1} of kanamycin were added to media as required.

1.2 Construction of plasmids and transformants

To assess the transcriptional activity of *Pgyl*, a reporter vector pDG was constructed from the plasmid pDR2 carrying a promoterless *xylE-neo* cassette. Briefly, a 324 bp

Table 1 Plasmids and strains used in this study

Plasmids or strains	Relevant features	Reference
	Plasmids	
pDR2	Double-reporter vector bearing promoterless *xylE-neo* cassette	[23]
pDG	pDR2 derivative with *Pgyl* in front of *xylE-neo*	This study
pSET152	*E. coli-Streptomyces* shuttle vector capable of integration into φ31 *attB* site in Streptomycetes	[26]
pSGR	pSET152 with *Pgyl* in front of *ccaR*	This study
pSCR	pSET152 with the intact *ccaR* with *PccaR* promoter	This study
	Strains	
	S. clavuligerus	
NRRL 3585	wild type, cephamycin C, CA and 5S clavams producer	NRRL
pDR	pDR2 integrated into NRRL 3585	This study
pDG	pDG integrated into NRRL 3585	This study
pSET	pSET152 integrated into NRRL 3585	This study
pSGR	pSGR integrated into NRRL 3585	This study
pSCR	pSCR integrated into NRRL 3585	This study
	E. coli	
DH5α	General cloning host	Invitrogen
ET12567/pUZ8002	Donor strain for conjugation between *E. coli* and *Streptomyces*	[25]

Table 2 Oligonucleotides used in this work

Names	Sequences (5′–3′) (restriction sites are underlined)	Purposes
Pgyl-1	AGTTAGATCTACGGCTTCGCAAGGGTGA	To clone *Pgyl* for the construction of pDG
Pgyl-2	AGTTACTAGTCCGCGACTCCTTTGTCCTTC	
Pgyl-3	AGTTTCTAGAACGGCTTCGCAAGGGTGA	To clone *Pgyl* for the construction of pSGR
Pgyl-4	AGTTGGATCCCCGCGACTCCTTTGTCCTTC	
ccaR-1	AGTTGGATCCCTTCACAAGGGGGACCGC	To clone a promoterless *ccaR*
ccaR-2	AGTTGCGGCCGCCGTTTCTTCAGCGTTGGTTCA	
ccaR-3	ATCGGATCCGCCCAGGTCCGG	To clone *ccaR* with *PccaR* promoter
ccaR-4	AGTGCGGCCGCCGTTTCTTCAGCGTTGGTTCA	
ccaRRT-1	GGTGACACTCGTGAAAGGTT	To amplify an internal fragment of *ccaR* for qRT-PCR
ccaRRT-2	AAACGGTCAGTGGTGGCTC	
claRRT-1	ATCTGCGGGACGAGACCTGG	To amplify an internal fragment of *claR* for qRT-PCR
claRRT-2	TGGGCGAGCCGAGACCTATC	
ceaS2RT-1	GTGTCGCCGCTGATGTCCT	To amplify an internal fragment of *ceas2* for qRT-PCR
cesS2RT-2	CGTTCGGGAAGATGTCGTG	
glpF1RT-1	TTGTCGTCGTCGGTATCGG	To amplify an internal fragment of *glpF1* for qRT-PCR
glpF1RT-2	ACGCCAGCTGGCGAAAGGG	
hrdBRT-1	AGGCCCGCACCATCCGTATC	To amplify an internal fragment of *hrdB* for qRT-PCR
hrdBRT-2	GGGTCATGTCGAGTTCCTTGGC	

DNA fragment upstream of the ATG start codon of *glpF1* (designated *Pgyl*) (Figure 3B) was amplified by PCR using Pgyl-1 and Pgyl-2 primers extracted from the genomic DNA of *S. clavuligerus* NRRL3585. To construct the pDG, the amplified fragments were digested with *Bgl* II/*Spe* I then ligated into the corresponding sites of pDR2 upstream of the *xylE-neo* cassette.

Plasmids pSGR and pSCR were constructed from pSET152. The former contains a copy of *Pgyl*-controlled *ccaR*, while the latter contains a copy of *PccaR*-controlled *ccaR*. To construct pSGR, *Pgyl* and the coding region of *ccaR* were obtained using primers Pgyl-3 and Pgyl-4, and the ccaR-1 and ccaR-2 primer pairs from the genomic DNA of *S. clavuligerus* NRRL3585, respectively. The PCR products were respectively digested with *Xba* I/*Bam*H I and *Bam*H I/*Not* I. The resulting two fragments were ligated into *Xba* I/*Not* I-digested pSET152 in a three-fragment ligation to generate pSGR. To construct pSCR, *PccaR* was PCR-amplified using ccaR-3 and ccaR-4 primers from the genomic DNA of *S. clavuligerus* NRRL3585 (Figure 2B). The PCR products were doubly digested with *Bam*H I/*Not* I and then ligated into the corresponding sites of pSET152 to generate pSCR. All plasmids were confirmed by digesting with appropriate restriction enzymes and sequencing.

The plasmids pDR2, pDG, pSET152, pSGR and pSCR were transferred into *S. clavuligerus* NRRL 3585 by conjugation via *E. coli* ET12567/pUZ8002. The transformants were designated *S. clavuligerus* pDR, pDG, pSET, pSGR, and pSCR, respectively.

1.3 RNA extraction and quantitative real time PCR (qRT-PCR) analysis

Mycelial samples used in transcriptional profile analysis were collected from *S. clavuligerus* grown in SA. mRNA samples were isolated and retained for subsequent qRT-PCR analysis. To minimize potential physiological shock to mycelial samples, the harvested cells were rapidly filtered onto cellulose acetate filters, frozen in liquid nitrogen and stored at −70°C.

Total RNAs were extracted using the RNeasy midi kit (purchased from Qiagen, Shanghai) according to the manufacturer's instructions. Quality and quantity of RNAs were examined by UV spectroscopy and confirmed by agarose gel electrophoresis. To remove chromosomal DNA contamination, each sample was treated with RQ1 RNase-free DNase (Promega, Beijing). Complete elimination of chromosomal DNA was confirmed by PCR. Following DNase treatment, RNA samples (800 ng) were reversely transcribed using Superscript III first-strand Synthesis System (Invitrogen, Beijing) following the recommended protocols. The reaction mixtures were prepared with SuperReal PreMix SYBR Green PCR master mix (Tiangen Biotech, Beijing), and qRT-PCR was performed in a 72-well rotor using Rotor-Gene Q system (Qiagen). The *hrdB* gene of *S. clavuligerus* (encoding the housekeeping sigma factor) was selected as the internal standard, and the primers used to amplify *glpF1*, *ccaR*, *ceas2*, *claR*, *hrdB* are listed in Table 2. At each time point, RNA isolation and subsequent analysis was performed in triplicate.

1.4 Assessment of *Pgyl* promoter activities in *S. clavuligerus*

To assess the promoter activities of cloned *Pgyl* (Figure 3B), the transformants bearing the *Pgyl-xylE-neo* reporter plasmid were evaluated for their XylE activities and kanamycin resistance. The XylE activities in cell-free extracts were

quantified by the following modified method [24]: Twenty milliliter aliquots of liquid cultures of the strains were centrifuged at 10000×*g* for 5 min. Supernatants were discarded and the cell pellets were washed once with distilled water then resuspended in 5 mL ice-cold sample buffer (100 mmol L^{-1} sodium phosphate buffer (pH 7.5), 20 mmol L^{-1} Na_2EDTA (pH 8.0), 10% (v/v) acetone). The cell suspensions were sonicated on ice (4×15 s, with 30 s intervals). Next, Triton X-100 was added to a final concentration of 0.1% (g/v) and the samples were placed on ice for 10 min. The cooled samples were centrifuged at 10000×*g* for 5 min and the supernatants were transferred to a fresh tube. In a pre-warmed (30°C) cuvette, 200 μL supernatants were added to 1 mL assay buffer (10 mmol L^{-1} sodium phosphate buffer (pH 7.5), 0.2 mmol L^{-1} catechol). The assay mixtures were incubated at 30 °C for 10 min. The absorbance at 375 nm was measured in a multi-mode micro-plate reader (Biotek). XylE activities were calculated as $50\times A_{375}$ mL^{-1} according to the standard protocol [27]. Relative activity is the XylE activity divided by that of the control strains. To measure kanamycin resistance levels, *S. clavuligerus* pDR, pDG and wild-type strain were streaked onto YD agar supplemented with kanamycin at different concentrations (50, 80 and 100 μg mL^{-1}). The plates were incubated at 28°C for 5 d before photographs were taken.

1.5 Analytical methods

To analyze CA production, 2 mL of culture broths were collected at regular intervals and centrifuged. The supernatants were filtered through a Millipore membrane (pore diameter 0.22 μm). Presence of CA was detected by observing the reaction of the filtered supernatants with equal volume of 3.0 mol L^{-1} imidazole solution (pH 6.8) at 37°C for 30 min [28]. The imidazole-derived samples were analyzed by HPLC using a reversed-phase column (Dikma Diamonsil C-18, 5 μm, 4.6 mm×250 mm) at 312 nm. The mobile phase consisted of 0.1 mol L^{-1} NaH_2PO_4 (pH 3.68) and 6.0% methanol (flow rate 1.0 mL min^{-1}). CA yields in *S. clavuligerus* strains were calculated by comparing measured levels with a calibration curve generated from CA standard solutions. Cell growth was quantified using the diphenylamine-colorimetric method as described previously [29].

2 Results

2.1 Assessment of transcriptional activities of *Pgyl* and *PccaR*

CA biosynthesis in *S. clavuligerus* requires co-expression of the *gyl* operon and *ccaR*. To determine whether both had been simultaneously expressed, the transcriptional profiles of *gylF1* and *ccaR* in the wild-type strain were analyzed by qRT-PCR. The glycerol-induced expressions of the two genes were similarly assessed. In the second assessment, *S. clavuligerus* NRRL 3585 was grown in SA medium, and glycerol induction activity was determined following addition of 1.5% glycerol at 60 h incubation. Samples collected at 36, 60 and 61 h were subjected to mRNA preparation and qRT-PCR analysis of *glpF1* and *ccaR*. The results suggest that the transcription level of *glpF1* was significantly enhanced (up to 5-fold) by glycerol (Figure 4A). In contrast, *ccaR* transcription was not significantly altered by glycerol (Figure 4B).

In addition, *Pgyl* was cloned and inserted upstream of the promoterless *xylE-neo* double reporter cassette in pDG. The promoter activity of this DNA fragment was evaluated by comparing the resultant XylE activities and kanamycin resistance levels of *S. clavuligerus* pDG with those of *S. clavuligerus* pDR and *S. clavuligerus* NRRL 3585. To quantify the enzyme activity of XylE, these three strains were grown in liquid YD media and sampled at 60 h incubation (Figure 5A). The XylE activity of *S. clavuligerus* pDG is observed to be much higher than that of both *S. clavuligerus* pDR and the wild-type strain, thus validating the promoter activity of *Pgyl*. In parallel with XylE activity,

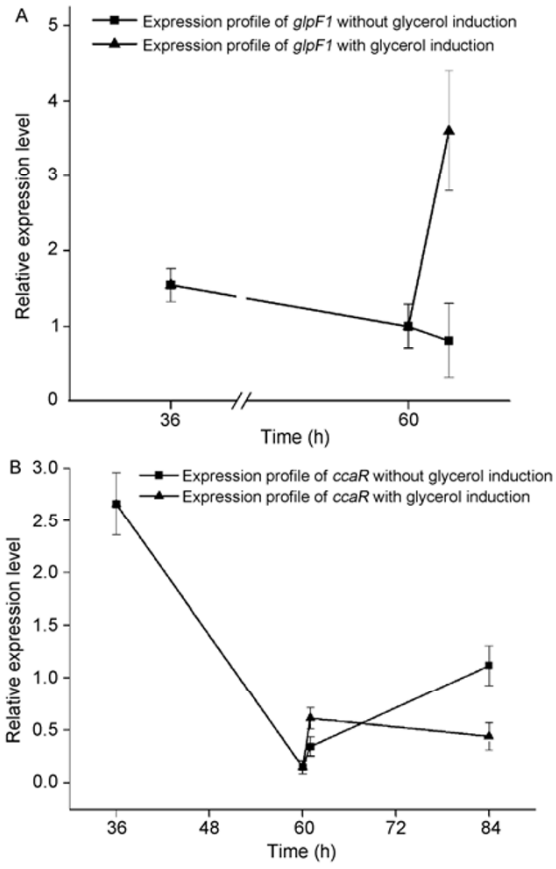

Figure 4 Transcription of *glpF1* and *ccaR* in wild-type *S. clavuligerus*. A, Transcriptional levels and profiles of *glpF1* in the wild-type strain cultured in SA media without glycerol (■) or with 1.5% glycerol added at 60 h incubation (▲). B, Transcriptional levels and profiles of *ccaR* in the wild-type strain cultured in SA media without glycerol (■) or with 1.5% glycerol added at 60 h incubation (▲).

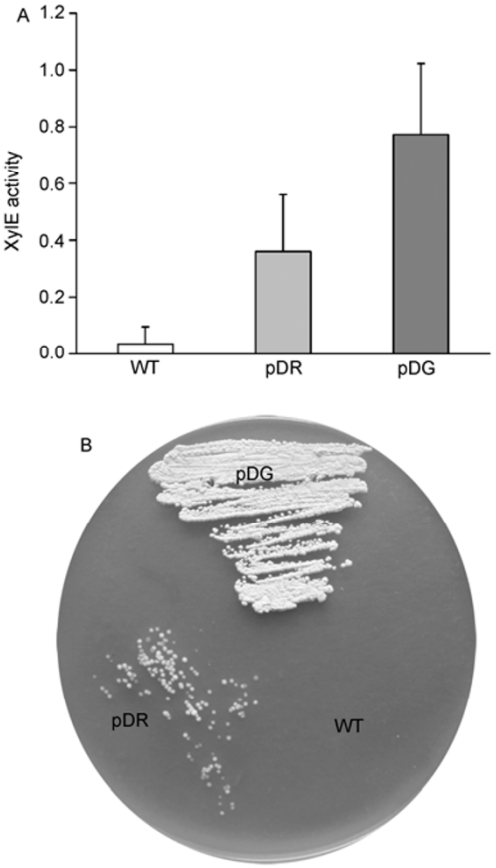

Figure 5 Evaluation of the promoter activity of *Pgyl*. A, XylE activity of wild-type strain and transformants (*S. clavuligerus* pDR and pDG). B, Kanamycin resistance exhibited by the wild-type strain and the transformants (*S. clavuligerus* pDR and pDG), cultured on YD agar containing 80 μg mL^{-1} kanamycin.

Pgyl confers kanamycin resistance to *S. clavuligerus* pDG at kanamycin levels exceeding 80 μg mL^{-1} (Figure 5B). This result is consistent with the enhanced XylE activity in the pDG-carrying strain. Taken together, these results confirm that the cloned *Pgyl* is an active promoter.

2.2 Evaluation of growth and CA production levels in engineered *S. clavuligerus* strains containing an extra copy of *ccaR*

The SARP family transcription activator *ccaR* regulates CA biosynthesis by directly controlling the transcription of *ceas2* and *claR* in *S. clavuligerus* (Figure 2A). In an attempt to coordinate glycerol utilization and CA biosynthesis, a promoterless *ccaR* was placed under *Pgyl* control to yield pSGR. This plasmid was then transfected into *S. clavuligerus*. The resulting strain, *S. clavuligerus* pSGR, was imbued with an extra copy of *ccaR* under the control of chromosomally-integrated *Pgyl*. The plasmids pSET152 and pSCR (the latter containing both *ccaR* and its promoter *PccaR* cloned in pSET152) were introduced into *S. clavuligerus* NRRL 3585 to obtain two control strains, *S. clavuligerus* pSET and pSCR. These strains were cultured in soybean flour media and their growth and CA titers were measured. While the growth of the pSGR strain was similar to those of the controls, its CA titer was increased by 3.19-fold relative to the pSCR strain (Figure 6A and B). These results suggest that coordination of glycerol utilization and CA biosynthesis by placing an extra copy of *ccaR* under *Pgyl* control is an effective strategy for increasing CA production.

2.3 Analysis of the transcriptional changes in *S. clavuligerus* pSGR

Having confirmed that CA production was significantly enhanced in *S. clavuligerus* pSGR, we seek the underlying reasons for this enhancement. To this end, we analyzed the transcriptional changes of *ccaR* as well as those of its regulatory targets (*ceas2* and *claR*). The transcriptional levels of *ccaR*, *ceas2 and claR* were determined by qRT-PCR, and were compared between *S. clavuligerus* pSGR, pSCR and the wild type. The strains were grown in soybean flour media supplemented with 1.5% glycerol and sampled at 24, 48 and 72 h incubation for mRNA preparation and qRT-PCR analysis. The transcriptional levels of all three genes in *S.*

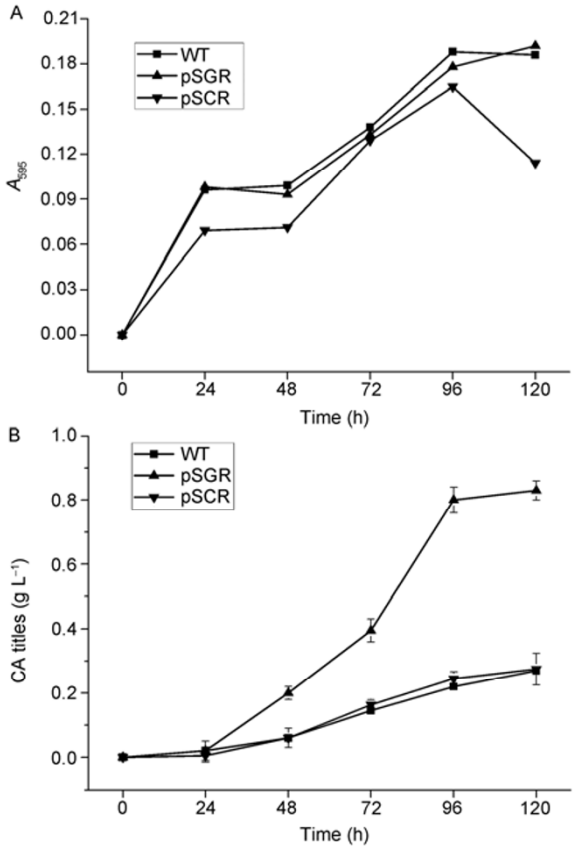

Figure 6 Growth (A) and specific CA production (B) of *S. clavuligerus* wild type (■), *S. clavuligerus* pSCR (▼) and *S. clavuligerus* pSGR (▲) in soybean flour media.

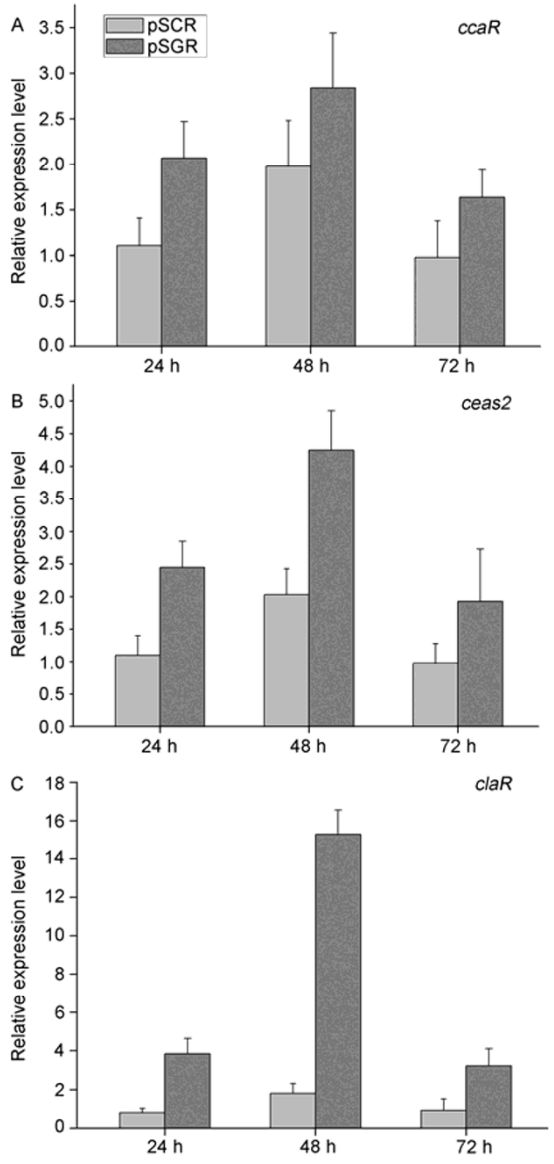

Figure 7 Relative transcription levels of *ccaR* (A), *ceas2* (B) and *claR* (C) in *S. clavuligerus* pSCR (gray) and *S. clavuligerus* pSGR (dark gray) at 24, 48 and 72 h incubation. Results are expressed relative to the transcription levels of the corresponding genes in *S. clavuligerus* NRRL 3585 (taken as 1).

clavuligerus pSGR were much higher than those in *S. clavuligerus* pSCR and the wild-type strain at all time-points (Figure 7). These results provide direct evidence that high CA production by *S. clavuligerus* pSGR is due to increased expression of *ccaR* under *Pgyl*, and the resulting elevation of *ceas2* and *claR* expression.

3 Discussion

The glycerol utilization operon is highly conserved in *Streptomycetes* (Figure 3A). In both *S. coelicolor* and *S. clavuligerus*, *gly* transcription is induced by glycerol and is mediated by a common regulator, GylR. The gene encoding GylR lies immediately upstream of the *gyl* operon, and GylR binds to an operator region immediately upstream of the *gyl* operon. In this work, the promoter activity of *Pgyl* was first verified and evaluated by a reporter method. That the native *gyl* operon is induced by glycerol was confirmed from the transcriptional profiles of *glpF1* in glycerol-supplemented culture (Figure 4A). Glycerol utilization and CA biosynthesis were then coordinated by introducing an extra copy of *ccaR* into the chromosome via the integration vector pSET152. This extra copy was controlled by the *Pgyl* promoter. The rationale behind this strategy was to place both *ccaR* and the *gyl* operon under the control of GylR, so that glycerol presence would stimulate expression of both. Ideally, this strategy should generate an engineered strain in which one copy of *ccaR* is regulated by glycerol induction, while the other remains intact and normally expressed. Once *ccaR* expression is under *Pgyl* control, the genes expressed during early and late stages of CA biosynthesis should be transcribed in the presence of glycerol, and transcriptionally coordinate with the C3-carbohydrate flux, thus increasing the titer of CA production.

CcaR is involved in the regulation of both CA and cephamycin C biosynthetic gene clusters (Figure 2A). The native promoter region of *ccaR* contains several *cis* regulatory sites, reflecting the complex regulation of its expression. Therefore, drastic modification of this region may dramatically affect the growth and antibiotic production of the altered phenotypes, as we have observed in the *argR* disruption mutant CZR [30]. Thus, we sought a more conservative approach by which to manipulate *ccaR* expression; creating an extra copy of *ccaR* controlled by chromosomally-integrated *Pgyl*. According to our results, this strategy significantly improves CA production levels. Indeed, the expression of not only *ccaR*, but also its target genes, was enhanced. However, whether the observed increase is due to coordinated expression of *gyl* and the CA gene cluster requires further analysis. Overexpression of *ccaR* using the *ermEp** promoter on either an integrative or a multiple copy vector has been shown to improve CA production [16]. In that work, as in our study, the native copy of *ccaR* was retained. Recently, Kurt et al. [17] constructed a *S. clavuligerus* mutant containing multiple copies of *ccaR* expressed from a *gyl* promoter (named P_{glpF1}). Following exposure to glycerol, cephamycin C levels and *ccaR* expression in the mutant were increased 6.1-fold and 5.1-fold, respectively, relative to the wild type; however, no effects on CA production were reported. Notably, the transcriptional profile of *ccaR* in *S. clavuligerus* pSGR (carrying an extra copy of *ccaR* expressed from *Pgyl*) was altered markedly from that of the wild type (Figures 4B and 7A). The expression profile of *ccaR* in *S. clavuligerus* pSCR (carrying an intact copy of *ccaR* controlled by *PccaR*) was also changed. However, CA production was not enhanced in this strain, possibly because the transcriptional strength was not

significantly improved.

This work presents a first attempt at coordinating CA biosynthesis and glycerol utilization in *S. clavuligerus*. Although several previous studies have selected *ccaR* as an overexpression target for increasing CA production, these have typically focused on strong promoters that increase *ccaR* expression. The strategy used in this work coordinates *ccaR* and *gyl* operon expression under a common glycerol-inducible promoter.

Ideally, CA production by the engineered strains carrying an extra copy of *ccaR* regulated by *Pgyl* should be further improved under glycerol-supplemented fermentation conditions. However, such an increase was not observed (data not shown). This suggests that *Pgyl*-regulated *ccaR* expression is not significantly stimulated by glycerol. Similar results were reported by Wang et al. [12] on cephamycin C production by *Pgyl*-regulated *ccaR*. They constructed an integrative vector carrying *ccaR* expressed from *gyl*P1/P2, the promoter of the glycerol utilization operon in *S. coelicolor*, to complement the *ccaR* disruption mutant in *S. clavuligerus*. They found that cephamycin C production by the complementation strain was independent of glycerol concentration, for reasons which are not clear.

Improving the production of secondary metabolites is a complex task. Coordinating the precursor supply with secondary metabolite biosynthesis can potentially improve product yield. However, such manipulation is much more difficult than simple overexpression of target genes, and the engineering design must be carefully evaluated and validated to ensure its success. By coordinating the expressions of *ccaR* and the *gyl* operon, we observed positive impacts on CA production, but the mechanistic details of the engineered expression of these genes have yet to be elucidated. To improve the design and optimize the performance of the engineered system, these issues must be carefully addressed.

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